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# TRANSPORT OF BOVINE MILK EXOSOMES IN HUMAN ENDOTHELIAL CELL

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# TRANSPORT OF BOVINE MILK EXOSOMES IN HUMAN ENDOTHELIAL CELL

by

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microRNAs (miRNAs) are small non-coding RNA which play an important role in gene regulation. Majority of miRNAs are encapsulated in exosomes thereby confer protection against degradation and transport across cell. Recent study suggests that 1) humans absorb microRNAs from cow's milk, 2) milk microRNAs are delivered to peripheral tissues where they alter the expression of human genes, and 3) endogenous synthesis of microRNAs does not compensate for dietary microRNA depletion in mice. Studies suggest that cow's milk exosomes can be transported across the intestinal mucosa by processes involving endocytosis and exocytosis. Here we tested the hypothesis that endothelial cells also transport milk exosomes. Using FM-labelled exosomes, the transport of exosomes in HUVECs can be assessed, i.e., the quantitatively most important fraction of exosomes in milk taken up by HUVECs. Exosome uptake followed Michaelis-Menten kinetics ($V_{max}$ = 0.057±0.004 ng exosome protein *x* 40,000 cells$^{-1}$ *x* hour$^{-1}$; $K_m$= 17.97±3.84 µg protein/200 µl media) and decreased by 80% when the incubation temperature was lowered from 37°C to 4°C, consistent with carrier-mediated transport. When exosome surface proteins were removed by treatment with proteinase K or transport measured in the presence of carbohydrate competitors, transport rates decreased by 30% to 50% compared with controls, consistent with a role of surface glycoproteins in

endothelial transport. Treatment with cytochalasin D caused a 50% decrease in transport, consistent with endocytosis. We conclude that human endothelial cells transport bovine exosomes by endocytosis and propose that this is an important step in the delivery of exosome cargo to peripheral tissues.

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# Chapter 1
# Literature Review

**Extracellular Vesicles (EVs)**

Extracellular vesicles (EVs) are term used to describe the diverse types of plasma membrane or endosomal vesicle origin (1). They appears in spherical form and composed by phospholipids and specific proteins which separates them from plasma membrane origin with size ranging from 30 nm to 1 μm (2-4). They are secreted by both eukaryotic and prokaryotic cell into the environment for cell-to-cell communication, protection, and exchange of genetic information (5).

In the beginning, EVs were considered as cellular debris, which is resulted from the membrane remodeling of apoptotic cells (6). Indeed, the first and well-known EVs was apoptotic bodies which is released during apoptosis. However, healthy cells also release vesicles which comprised by large and small vesicles (7-10). Some EVs are derived from the shedding of plasma membrane which latter known as shedding vesicles or microvesicles (11), whereas other EVs are derived from the exocytosis process of multivesicular bodies (MVB) which known as exosome (12). These two types of EVs are majorly found to be released by the cell into the plasma as well in other biological fluids such as semen, saliva, urine, breast milk, cerebrospinal fluids, bronchoalveolar and lavage fluid, amniotic fluid, and malignant ascites (13-18).

Perhaps the first experiment regarding the presence of EVs was dated back in 1967 when Wolf (19) reported phospholipids rich small vesicle ranging from 20 to 50 nm from the platelet termed “platelet dust” that contains the platelet factor 3 for blood coagulation. Ultracentrifugation removed the small vesicle and caused a prolonged blood coagulation. The presence of small vesicles were confirmed later in fetal bovine serum, nerve tissue, healthy cell and cancerous cell with unknown biological function (7-9). In

the early 1980s, two independent researchers have identified the function of this small vesicle for transporting transferrin and its receptor during reticulocyte maturation (10, 20). Further electron microscopy identified that the small vesicle was formed by inward budding of plasma membrane into intracellular endosomes followed by exocytosis of late endosome or multivesicular endosomes (MVBs) (10, 21). Term "exosomes" than had been used to characterize this small vesicles produced by mature reticulocytes which carry the transferrin receptor without the presence of cytosolic enzyme that can be isolated using centrifugation technique (12, 22). Noted that the term exosome used here should be distinguished from the RNA degrading complex in the cytosol (23).

However, these findings were not appreciated enough until Raposo et al. (24) reporting the presence of major histocompatibility complex (MHC) class II in exosome-like vesicle produced by antigen presenting cell. This result indicated that exosome is not only produced by the reticulocytes, but also from other cells such as B cell. This finding was confirmed by several studies that cancer and other normal cells also produce exosome vesicle that carry functional enzyme (25), mRNA (26) and microRNA (27, 28). Since then, large body of articles have identified numerous EVs isolated from different cell with distinct functional therapies such as cardioprotection (29), drug delivery (30), stroke therapy (31), and gene therapy (32).

**Classification of Cell Derived EVs**

Although several cell derived EVs have been well characterized in numerous studies, however there is no consensus regarding the nomenclature and characteristic of cell derived EVs (33, 34). This condition led to the confusion on determining the cell derived EVs. For instance, one can use the term “ectosomes” (35, 36), “exosome-like vesicles” (37, 38), “argosomes” (39), “tolerasomes” (40, 41), “prostasomes” (42), “cardiosomes” (43) or “vexosomes” (44) to explain small vesicle with size <100 nm. The term of microparticles and microvesicles have been used collectively by numerous authors to describe direct, vesicular form of membrane shed vesicles with size >100 nm (45). Moreover, exosomes can be defined in different ways, i.e., the exfoliated plasma membrane, which has the plasma ecto-enzyme activity (9), or the vesicles that formed by inward budding of limited plasma membrane into endosome to form MVBs followed by exocytosis process of MVBs after fusion with plasma membrane (22). In order to make the classification clear, the term microvesicles (MVs) will be used instead of microparticle to explain vesicle larger than 100 nm.

**Table 1.1**. Characteristics of different types of cell derived EVs*)

| Feature | Exosome | Microvesicles | Membrane Particles | Ectosomes | Exosome-like particle | Apopto-tic bodies |
|---|---|---|---|---|---|---|
| Size (nm) | 30-100 | 100-1,000 | 50-80 | 50-200 | 20-200 | 50-5000 |
| Density (g/ml) | 1.13-1.19 | ND | 1.03-1.07 | ND | 1.12-1.18 | 1.16-1.28 |
| Mechanism of generation | Exocy-tosis of MVBs | Budding/blebbing of plasma membrane | Budding/ blebbing of plasma membrane | Budding/ blebbing of plasma membrane | ND | Release from dying/ap optotic cells |
| Morphology | Cup-shaped | Irregular shape | Round | Bilaminar round | Cup-shaped | Heteroge neous |
| Origin | Endoso-mes | Plasma membrane | Plasma membrane | Plasma membrane | Internal compartment ? | ND |
| Protein markers | Tetra-spanin (CD63, CD9, CD81), LAMP, Alix, TSG101 | Annexin V, integrins, selectins, CD40 ligands | CD133 | CD59, CR1-related enzyme | TNFRI | Histone |

*) Adapted from (13, 35, 45-50). ND, not defined.

Based on literature, there are several categories of cell derived EVs. Some classified cell derived EVs into two classes (51), three classes (1, 45, 47, 49), four classes (48) or more (46). Characteristics of cell derived EVs are reviewed in Table 1.1. These discrepancies in classification of cell derived EVs are caused by many factors, i.e., isolation procedure, size, density, morphology, lipid and protein composition as well cellular origin (33, 48). Thus, to mediate the ambiguous term in cell derived EVs research, International Society of Extracellular Vesicles (ISEV) has proposed some criteria to represent the minimal characterization of EVs. These including: 1) the presence or absence of some protein marker, which can be detected by western blot, flow cytometry, or mass-spectrophotometry, 2) characterization of single vesicles should be performed by using electron microscopy in wide area accompanied by size distribution measurement, 3) presence of controls in the studies of the functional activity of EVs (34).

**Exosome**

Exosome is small, nano-sized vesicle with diameter of 30-100 nm and density of 1.13-1.19 g/mL (50). It is secreted by normal or cancerous cell in spontaneous or inducible processes (9, 52, 53). It is present in biological fluid such as plasma, saliva, urine, breast milk, bronchoalveolar lavage fluid, amniotic fluid, cerebrospinal fluid, and malignant ascites (13, 14, 17, 54).

Exosome term was first determined by Trams et al (9) as a vesicle derived from the plasma membrane although the term was started to change two years afterwards when two independent researchers, Harding et al (10) and Pan et al (20), discovered a small vesicle formed from inward budding of late endosomes. This small vesicle contains transferrin receptor, which add another route for transferrin receptor recycle during maturation of red blood cells (12). Electron microscopy further revealed the formation of intraluminal vesicles (ILV) formed by invagination or budding from limited membrane of endosome, which will fuse with plasma membrane to release small vesicle through exocytosis process (10, 21). Small vesicles, which are released from MVBs, were later termed “exosome” (22) and later were confirmed in B-cell by Raposo et al (24). Since then, term exosomes is used to describe the small vesicles formed by the inward budding of endosomes (Figure 1).

Exosomes harbor protein, lipid, carbohydrate and nucleic acids such as miRNA, small non-coding RNA and mRNA which derived from their cellular origin (55). These components, particularly miRNA, reflect the functionality to the host cell and posse molecular signature from their cellular origin (38, 47, 56-58). Therefore, it was thought

that molecular characterization of exosome exert potential biomarker of the disease such as cancer.

The protein content in exosome comprised by protein involved in MVBs generation such as membrane transport and fusion protein (e.g., Rab, annexins, flotillins), MVB biogenesis (e.g., ESCRT family: Alix, TSG101), cytoskeletal protein (e.g., actin, tubulin, syntenin, and moesin) and tetraspanin (e.g., CD63, CD9, CD81, CD82) although other proteins which is not related to the MVBs generation also present in exosome such as metabolic enzymes, heat shock protein, ubiquitin, and protein important for signal transduction, apoptosis and protein synthesis (46, 59, 60). Protein contents in exosomes are reviewed in Table 1.2.

The lipids of exosome comprised by cholesterol, phospholipids, sphingolipids, ceramides and lipid raft molecule (1, 46, 47). The carbohydrates in exosome is enriched in mannose, polylactosamine, α-2,6 sialic acid and complex N-glycans, which is important for protein recruitment in exosome (61-63). All of these components discrete exosomes from other organelle or plasma membrane (47). Currently, all of the protein and lipid components of exosomes are available online from three databases; EVpedia, Exocarta and Vesiclepedia (64, 65).

Exosome also carry nucleic acids which can be translated into functional protein or regulate the activity of gene (26, 66, 67). Several RNA families such as messenger RNA (mRNA), microRNA (miRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), piwi RNA (piRNAs), mitochondrial RNA (mitRNA) and long non-coding RNA present in exosome (60, 68-70). The presence of nucleic acids, especially the miRNA, are thought to be a reliable marker for exosome due to the equal level of miRNA in exosome

compared with the parental cell (15, 70, 71). However, identification of miRNA in exosome is challenged due to the low level of miRNAs in exosome compared to their cell origin (72). In addition, recent studies also suggest that other proteins; e.g., the HDL and argonaute, also transport miRNA which limit the potential use of miRNA detection as a marker of exosome (73-75).

Based on the first discovery of exosomes, the exocytosis process of MVBs is the general process of exosomes biogenesis. The engulfment of plasma membrane by clathrin or non-clathrin coated pits resulting in early endosome was the first pathway in exosome biogenesis. The formation of early endosome was followed by multivesicular bodies (MVBs) development through trans-golgi network protein interaction, i.e., endosome sorting complex required for transport (ESCRT), resulting in the formation of intraluminal vesicle (ILV). The ESCRT protein family is also important for the fate of MVBs (76). The fusion of MVBs with the plasma membrane resulted in the releasing of exosomes. This mechanism is first confirmed in red blood cell, which was also established in dendritic cells (77).

However, there are other exosome biogenesis pathways, which are ESCRT-independent process. This pathway rises as silencing the production and activity of TSG101 or ESCRT complex did not hamper the production of exosome in some type of cells (53, 78, 79). For instance, silencing the production of CD69 protein in melanocyte has caused significant reduction in exosome biogenesis (80) while inhibiting ceramide production in oligodendroglial cell has resulted in lower production of exosomes (81). These results indicated the ESCRT-independent pathway for exosome biogenesis as well

the cellular specific pathway for exosome biogenesis, which may explain the variety on molecular signature in exosomes (82).

Upon releasing into the plasma or serum, recent direct and indirect evidence reported that exosomes are taken up by the cell through several proposed mechanisms in order to transfer the cargo into recipient cells. Studies showed that exosomes uptake is endocytosis-dependent process as indicated by fluorescence microscopy method (83-88). In addition, other types of endocytosis process, i.e., phagocytosis and micropinocytosis, have been reported (89, 90). However, exosomes are also transported by direct fusion with plasma membrane of the cell, which suggesting the endocytosis-independent pathway for cellular internalization of exosomes (91).

**Microvesicles**

Microvesicles, also known as shedding vesicles, microparticles, ectosomes or membrane particles, is vesicles produced by outward budding and fission of plasma membrane (51, 92). They are found in most of biological fluids as well in atherosclerotic plaque (93, 94). Unlike exosomes, this small vesicles has heterogeneous shape, larger in diameter (from 100 nm up to 1 μm) and float in sucrose gradient with density of 1.032-1.07 g/mL, slightly lower than exosomes (46, 48, 92).

Microvesicles were first characterized by Wolf (19) as “platelet dust” that contain platelet factor 3 for blood coagulation. The vesicles were comprised by phospholipid rich vesicles and secreted by most type of cells in the body including circulating cells, vascular cells, and cardiomyocytes (95). Stress, hypoxia, apoptosis, inflammation, complement attack, bacteria lipopolysaccharides (LPS), intercellular calcium elevation,

senescence and platelets activation have been reported to stimulate the release of microvesicles (96-102). Thus, microvesicles play important role in coagulation (103), atherosclerosis development (104), tumor invasion and metastases (94), immunity and inflammatory modulator (105-107).

Due to the plasma membrane origin, microvesicles contain several proteins and lipid from plasma membrane origin. In addition, microvesicles also acquired some protein from plasma or endocytic compartment, which indicated sorting protein process in microvesicles (99, 110). Although, they are originated from plasma membrane of the cells, protein and lipid component of microvesicles, as well the cargo content of microvesicles, are depend on several factors; i.e., cellular origin, cellular stage (e.g., resting, stimulated), and the stimulation agent (111). For instance, stimulation of platelet with complement complex C5b-9 resulted in platelet-derived microvesicles enriched in C9 neoantigen of the C5b-9 complex and α-granule-derived-coagulation factor V (or Va) (112). In addition, stimulation with this agent produced platelet-derived microvesicles that did not bind with fibrinogen (113).

Although it is cleared that microvesicles was originated from plasma membrane of the cells, formation and released of microvesicles from plasma membrane is poorly understood. It is hypothesized that activated cells will cause an elevation of calcium level in the cytosol, particularly in site of vesiculation, will cause activation of kinases and calpain as well inhibition of phosphatases, which in turn will break the membrane cytoskeleton, releasing microparticles to extracellular compartment of the cells (98). Prior to membrane breakdown, it is hypothesized that non-secretory exocytic vesicles will accumulate into the plasma membrane, causing the budding of plasma membrane (51).

This event is characterized by the presence of desmoyokin and annexin 2, a marker of exocytic organelle, in microvesicles (111). It is also reported that budding of microvesicles from plasma membrane requires two important proteins: endosomal sorting complex required for transport (ESCRT) and arrestin domain-containing protein 1-mediated microvesicles (ARMMs) (114, 115). Inhibition of ARMMs along with ESCRT inhibits the release of microvesicles from plasma membrane, which indicated the main function of these two proteins in mediating the release of microvesicles from plasma membrane (116).

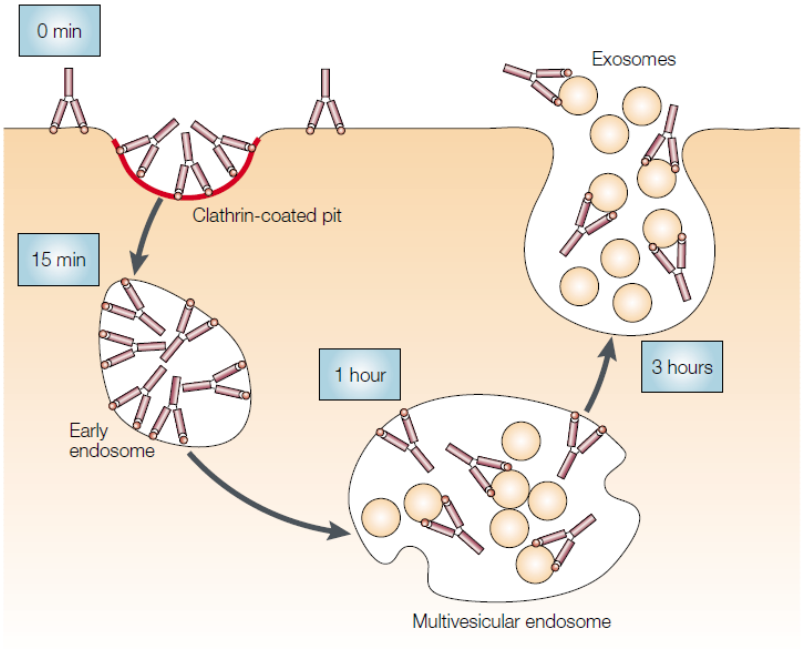


**Figure 1.1**. Formation of exosome from multivesicular endosome. A limited plasma membrane is engulfed into the early endosome where it will mature into multivesicular endosome with the formation of intraluminal vesicle (ILV) derived from late endosome. The multivesicular endosome then fused with the plasma membrane to release small vesicle containing transferrin receptor. Adapted from Thery et al (46).

**Table 1.2**. Protein and glycolipid content of exosomes*)

| Protein Function/Class | Protein |
|---|---|
| Antigen presentation | MHC Class I/II, CD86 |
| Adhesion molecules/targeting | Milk Fat Globule-Epidermal growth factor VIII (MFG-E8 or lactadherin), integrin (α3, α4, αM, αL, β1, β2), tetraspanin (CD63, CD9. CD37, CD53, CD81, CD82) |
| Membrane transport and fusion | RAP1B/RABGDI, Rab 7, Rab 2, Annexins (Annexins 1-7), dynamin, syntaxin, AP-1, Arp2/3, SNAP |
| Heat-shock protein | HSC70, HSP84/90 |
| Cytoskeletal protein | Actin, cofilin, tubulin, moesin, rodixin, advilin, vimentin, talin, CAP1, ezrin |
| Raft-associated protein or glycolipids | Flotillin, CD55, CD59, GM1, GM3, Gi2α, cholesterol, stomatin |
| Enzymes | Pyruvate kinase, alpha enolase, ATPase, glucose 6-phosphate, isomerase, peroxiredoxin 1, aspartate amino transferase (ASAT), fatty acid synthetase, ATP citrate lyase |
| MVB formation | Alix, TSG101, Gag |
| Signal transduction | Erk2, Fyn, RhoA/C, catenin, syntenin |

*) Adapted from (108) and (109)

Upon the release into extracellular compartment, microvesicles are taken up by the cells with half-life around 10 minutes in the plasma (117). Study indicated that microvesicles are taken up by the cells through endocytosis and phagocytosis pathway upon binding with specific receptor in the cells (118). Developmental endothelial locus (Del)-1 is important protein that recognize the phosphatidylserine in microvesicles and mediates the endocytosis process of microvesicles through binding with integrin, αvβ3, on cells (119). The lactadherin, also known as milk fat globule-epidermal growth factor 8, is mediated the phagocytosis process of microvesicles upon binding with integrin of macrophages (120). In addition, other proteins are reported to mediate the uptake of microvesicles; growth arrest specific 6 (GAS6), β2-Glycoprotein I, P-selectin (117).

**Apoptotic Bodies**

Apoptosis, also known as programmed cell death, is a major mechanism of cell death, both in cancer and normal cell, indicated by the release of membrane vesicles called apoptotic bodies or apoptosomes (6). The apoptosis process begins by activation of caspase protein in response to ligation of the death receptor in the cell membrane (CD95/FAS, TNFR1, TRAILR) (121). Activated caspase will cleave certain regions of genomic DNA, forming oligonucleosome ladder, followed by migration of oligonucleosome into the cytosol due to increased permeability of nuclear pores (122, 123). Upon exiting the nucleus, the oligonucleosome will migrate into plasma membrane, forming “blebs” at the plasma membrane (124, 125). It is reported that rho effector protein I (ROCK I) is cleaved by caspase and promote the formation of apoptotic blebs

during apoptosis (126, 127). The blebs will contain cellular organelle, which will be cleared through phagocytosis by macrophage (128).

Although apoptotic bodies can be distinguished from others EVs; e.g., exosomes and microvesicles, by size (500-4000 nm in diameter), study suggests the presence of cellular organelle in small vesicles with diameter of 50-500 nm, which indicating the apoptotic bodies (129). In addition, some proteins present in other EVs such as epithelial cell adhesion molecules (EpCAM), CD63, CD81, which is found in most of extracellular vesicles including apoptotic bodies, and annexin V, which is detected in microvesicles and apoptotic bodies (130, 131). The similarity in size and protein composition between apoptotic bodies and others EVs have added the difficulties in characterizing EVs from the sample. Moreover, there is no established protocols for isolating apoptotic bodies. One method that has been developed to distinguish apoptotic bodies from other EVs is RNA analysis. Based on this technique, apoptotic bodies majorly composed by ribosomal RNA, which is lacked in others EVs such as exosome and microvesicles (131).

Similar with other EVs, apoptotic bodies are cleared or taken up by the cells to mediate transfer of genetic between cells. Phagocytosis by the macrophage is the major route for apoptotic bodies' clearance during normal development (132). The clearance process of apoptotic bodies consisted of 4 distinct steps: accumulation of phagocytes at the site where apoptotic cells are located through "find me" signals, recognition of apoptotic bodies through "eat me" signals and their cognate receptors, engulfment by macrophages through signaling pathways that regulate the cytoskeletal rearrangement of macrophage, digestion of engulfed apoptotic bodies within macrophage (133, 134). The presence of thrombospondin, complement protein C3b, and annexin V in apoptotic

bodies are believed to guide the macrophage to eat the apoptotic bodies, which serve as a protein marker for apoptotic bodies (125, 132, 133). Internalization of apoptotic bodies into targeted cells has been reported to promote several functions such as anti-inflammatory properties (135, 136), viral transfer (137), endothelial cell repair (130), as well as cancer metastases (138).

**Biological Function of Cell derived EVs**

The presence of nucleic acids such as DNA, RNA, miRNA as well long non coding RNA in EVs has risen the importance of EVs for cell-to-cell communication (139). Indeed, the nucleic acid, particularly RNA, in EVs can be translated by recipient cells to promote function such as transcription termination (140) and lipid synthesis (141). Moreover, it is reported that most of detectable microRNA present in biological fluid such as serum, saliva and breast milk is encapsulated in EVs, particularly in exosomes (27). EVs exert potential protection against harsh environment and enzymatic degradation (142), which contribute to the transport and genetic transfer into recipient cells through local or systemic circulation in endocrine-like pathway (82).

Study suggest that mRNA and microRNA-containing EVs can be transmitted across species. Using mouse dendritic cells, Valadi et al (26) have successfully demonstrated that mRNA from mouse dendritic cells can be translated into functional protein in human mast cells. In addition, Sun et al (143) reported the immune-related miRNAs from cow's milk can influence gene transcription in human macrophage cells resulting in macrophage differentiation. Recent study conducted by our lab (67, 144) also

supported the notion that microRNAs present in cow's milk and egg EVs can be exported into human blood and affect gene expression in human body.

In addition, protein, enzyme and lipid components in EVs have been proposed to mediate several physiological or biochemical process in the body, i.e., cellular development, differentiation, and immune function. For instance, exosomes account for transferrin receptor transport from reticulocytes into the blood during red blood cells maturation (10, 145). In addition, Skokos et al (146) demonstrated that exosomes released from mast cells are able to induce maturation of immature dendritic cells (DC) by its ability in antigen presentation to T cells. Further studies indicated that the ability of antigen presenting of DC to T cells is mediated by exosomes released from mature DC (147, 148).

**MicroRNA (miRNA)**

microRNA (miRNAs) is small, ~21 nucleotides long, non-coding RNA that plays an important role in gene regulation, both in animals and plants, by binding to untranslated region of mRNA, resulting in translation inhibition or mRNA degradation (149). It was first discovered in *C. elegans* as a non-coding RNA, produced by lin-4 and let-7 genes, that binds to 3-untranslated region of lin-14, lin-28, lin-41, lin-42 and daf-12 mRNA (150, 151). Since then, let-7 RNA homolog has been identified in a wide range of animal species, e.g., vertebrae, ascidian, hemichordate, mollusk, annelid, and arthropod (152). This small, single stranded RNA was named miRNA and regulates as much as 60% of mammalian genes (153-156).

Since the discovery of miRNAs, other small RNAs have been characterized in animals, plants, and fungi, which including small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) (157, 158). They have distinct characteristic, although have similarities particularly in post-transcriptional modification. The characteristic between miRNA, siRNA and piRNA are reviewed in Table 1.3.

**MicroRNA Biogenesis, Maturation and Molecular Mechanism**

Study suggest that miRNAs are encoded in the nucleus. Most of the miRNAs in human, around two third, are encoded in the intron region of protein-coding genes as well in long non-coding transcript (159). This condition let to the assumption that miRNAs are transcribed along with the host genes using RNA polymerase II (160, 161). However, Bortolin-Cavaille et al (162), reported the introgenic independent pathway of protein coding genes for miRNAs biogenesis. They reported that miRNAs in C19MC are

transcribed from intron region of non-protein-coding genes. In addition, studies suggest the role of exonic region of non-protein-coding genes for miRNA production (163, 164). Thus, miRNA can be encoded by three genomic regions in the nucleus; 1) intronic region of protein-coding genes, 2) exonic miRNA region in non-protein-coding genes and or 3) intronic region of non-protein-coding genes.

**Table 1.3.** Characteristic of miRNA, siRNA, and piRNA*)

| | miRNA | siRNA | piRNA |
|---|---|---|---|
| Origin | miRNA gene (exon) or DNA intron (endogenous) | Long double stranded RNA (endegonous and/or exogenous) | Repetitive elements such as retrotrans-posons, DNA trans-posons, and the Su(Stellate) locus |
| Configuration | Single stranded RNA | Double stranded RNA | Single stranded RNA |
| Length | 19-25 nucleotides | 21-22 nucleotides | 21-33 nucleotides |
| Complementarity to target mRNA | Not exact | 100% perfect match | 100% perfect match |
| Action | Cleveage of mRNA and/or translation inhibition | Cleavage of mRNA | Cleavage of mRNA and recruitment of DNA methylation |

*)Adapted from (155, 165-168).

Biogenesis of miRNAs start with the translation of genomic region of miRNA in the nucleus. There are two enzymes: RNA polymerase II or RNA polymerase III, which mediate the transcription of miRNA (161, 169). The resulting product are called primary precursor of miRNA (pri-miRNA) that comprised by more than 1 kbp nucleotides with a double stranded stem of ~33 bp, a terminal loop and two flanking unstructured single-stranded segments (160, 170). The pri-miRNA is cleaved by microprocessor complex (nuclear RNAse III Drosha/Pasha and DGCR8) to release ~65 bp hairpin-shaped nucleotides called precursor miRNA (pre-miRNA) (171-174). The resulting pre-miRNA will be exported outside the nucleus using exportin-5, which require the Ran-GTP cofactor (175, 176). In addition, the binding of pre-miRNA with exportin-5 also protects pre-miRNA from degradation (177).

In the cytosol, pre-miRNA will subsequently cleave by another RNAse III nuclease called dicer to produce mature, 22 nucleotides miRNAs (178, 179). Dicer will recognize and bind to pre-miRNA in the 2-nt 3`-overhang in pre-miRNA through the PAZ domain in dicer (180-182). The binding of pre-miRNA with dicer will create a complex termed miRNA duplex, which will release a single stranded, mature miRNA. The mature miRNA will form a complex with Argonaute (Ago) protein while the other miRNA duplex will be degraded (170).

Argonaute protein and its homolog are ~100 kDa proteins, which contain both PIWI and PAZ domain (183, 184). When the mature miRNA, as well siRNA, forms a complex with the argonaute, the resulting complex is referred to RNA-induced silencing complex (RISC) (185). The complex will identify the mRNA target based on the complementary bases (perfect or nearly perfect) and cleaves the mRNA at a site near the

middle of miRNA/siRNA complementarity (186, 187). In addition, it was proposed that the argonaute-bound miRNA complex could repress the translation of mRNA (188, 189). The mechanism in which miRNA-argonaute protein complex inhibits protein translation lies in the sequence motive in argonaute protein that can bind with functional 7-methyl-guanine ($m^7G$) cap of mRNA (190). Once the argonaute form a complex with $m^7G$ cap of mRNA, this cap is not accessible for elf4E to initiate the translation process of mRNA, resulting in translation inhibition of mRNA (191, 192).

The translation inhibition mechanism of argonaute-miRNA complex is also postulated due to the ability in the formation of "pseudo-polysomes", large EDTA sensitive mRNA-protein (mRNP) assemblies (193). It is hypothesized that formation of pseudo-polysomes was caused by protein interaction between GW182 with argonaute protein, which causing recruitment of 80S ribosomes to mRNA (194-196). This polysomes lack of translation machinery and reported to initiate mRNA degradation (197, 198). Biogenesis, maturation and molecular mechanism of miRNA are reviewed in Figure 1.2.

**MicroRNA in Health and Disease**

Due to the important role of miRNAs in regulating gene expression in the cells, miRNAs have been associated with health or disease progression. For instance, miR-29b has been reported for bone remodeling through its effect in genes regulation in osteoclast and osteoblast differentiation (199, 200) while miR-200 subfamily (miR-200b/200c/429) has been reported to inhibit cancer metastasis in hepatocellular carcinoma (201-203). Thus, alteration in miRNAs level may impact health and disease progression of the host

and can potentially be used as diagnostic tools in various disease state such as carcinoma (204, 205), osteoporosis (206), type 2 diabetes mellitus and obesity (207), as well in cardiovascular disease (208).

Although miRNAs are considered endogenous; e.g., miRNAs synthesized by the cells will be used by the cells to regulate the gene expression inside the cells, recent studies suggest that dietary miRNAs may contribute to the miRNAs body pool. Indeed, miRNAs present in food can be transferred into human through dietary means. The first study regarding the importance of dietary miRNAs in body pool miRNAs and the effect in human health was demonstrated by Zhang et al (210). In this study, they reported that rice miRNA, osa-miR-168a, is detected in the mouse and human sera and inhibits mRNA expression of LDL-receptor, thereby reducing LDL removal from the blood. However this study was challenged by several findings regarding the low bioavailability of plant miRNA in the blood (211-213). Interestingly, miRNAs present in milk and egg have been reported to be absorbed in biologically meaningful amount and affect human gene expression both in vivo and in vitro (67, 144). The discrepancies of the result was probably caused by the encapsulation of miRNAs in EVs that protect miRNAs from enzymatic degradation and harsh environment as well for miRNAs transportation across species (142, 214, 215).

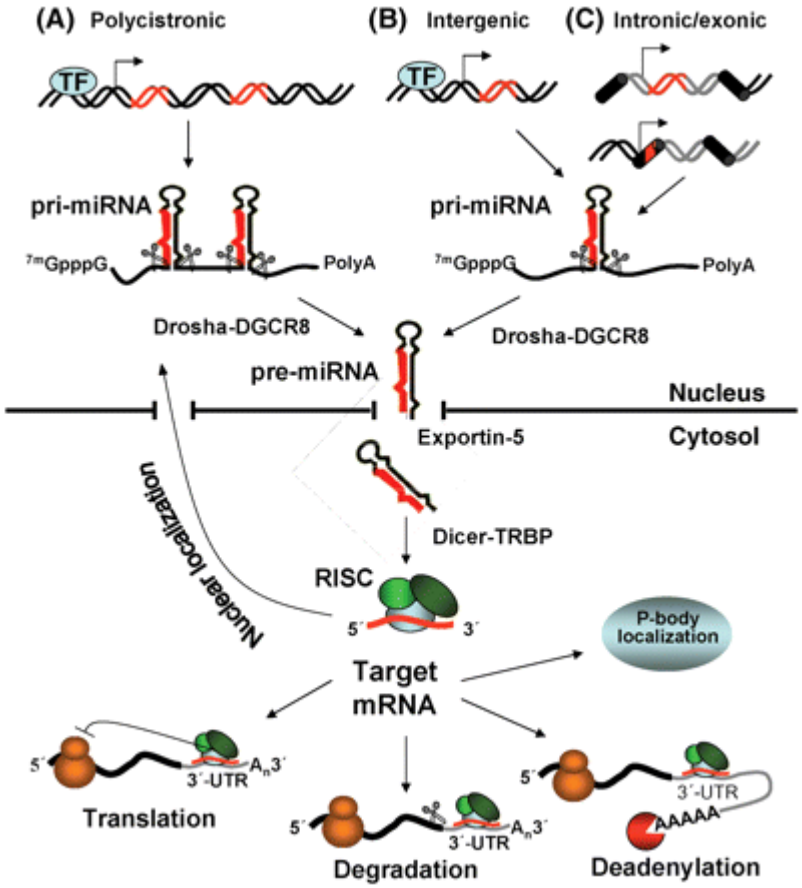


**Figure 1.2**. Biogenesis, maturation and molecular mechanism of miRNA. In the nucleus, miRNA is transcribed using RNA polymerase II/III to produce pri-miRNA. The pri-miRNA will subsequently cleave by Drosha/Pasha to produce pre-miRNA and exported to cytoplasm using exportin/RAN-GTPAse. In the cytosol, pre-miRNA will be cleaved by Dicer, releasing mature, single stranded miRNA. Mature miRNA will form a complex with argonaute protein calling RNA induced-silencing complex (RISC). Several mechanisms and possible pathways have been proposed, e.g., inhibition of translation or mRNA degradation. Adapted from (209).

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# Chapter 2

# Transport of Bovine Milk Exosomes in Human Endothelial Cells

**Human endothelial cells transport foreign exosomes from cow's milk by endocytosis**[1,2]

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**ABSTRACT**

MicroRNAs (miRNAs) can be synthesized endogenously and cause gene repression. Encapsulation of miRNAs in exosomes confers protection against degradation and a vehicle for shuttling between cells and tissues, and cellular uptake by endocytosis. Exosomes can be found in biological fluids and foods including milk. Evidence suggests that humans absorb cow's milk exosomes and deliver the microRNA cargo to peripheral tissues, consistent with gene regulation by nucleic acids across species boundaries. There is strong evidence that milk exosomes may cross the mucosa without re-packaging in mice. Here, we tested the hypothesis that human vascular endothelial cells transport milk exosomes by endocytosis, as a crucial step toward delivery of dietary microRNAs to peripheral tissues. Studies were conducted using human umbilical vein endothelial cells and fluorophore-labeled exosomes isolated from cow's milk. Exosome uptake followed Michaelis-Menten kinetics ($V_{max}$ = 0.057±0.004 ng exosome protein *x* 40,000 cells$^{-1}$ *x* hour$^{-1}$; $K_m$= 17.97±3.84 µg protein/200 µl media) and decreased by 80% when the incubation temperature was lowered from 37°C to 4°C, consistent with carrier-mediated transport. When exosome surface proteins were removed by treatment with proteinase K or transport measured in the presence of carbohydrate competitors, transport rates decreased by 30% to 50% compared with controls, consistent with a role of surface glycoproteins in endothelial transport. Treatment with cytochalasin D caused a 50% decrease in transport, consistent with endocytosis. We conclude that human endothelial cells transport bovine exosomes by endocytosis and propose that this is an important step in the delivery of exosome cargo to peripheral tissues.

**Keyword**: endocytosis; endothelial cell; extracellular vesicles; milk exosomes; uptake

**INTRODUCTION**

MicroRNAs (miRNAs) are small non coding RNA, which are encoded by their own genes, or introns and exons of long nonprotein-coding transcripts (1-3); miRNAs may silence genes via destabilizing of messenger RNA or preventing translation of mRNA (4, 5). The online database *miRBase,* release 21, lists 1881 "high confidence" human miRNAs (6, 7), and about 60% of the miR-binding sites in the human genome are evolutionary conserved (8). Gene regulations by miRNAs has been implicated in numerous physiological (9) and pathological (10) conditions in humans.

Until recently, miRNAs have been considered endogenous regulators of genes, i.e., miRNAs synthesized in a given organism regulate the expression of genes in that host. In a recent publication we have refuted this paradigm and provided strong evidence that 1) humans absorb biologically meaningful amounts of miRNAs from nutritionally relevant doses of cow's milk, 2) milk miRNAs are delivered to peripheral human tissues, 3) physiological concentrations of milk miRNAs affect human gene expression *in vivo* and in cell cultures, and 4) endogenous synthesis of miRNAs does not compensate for dietary miRNAs deficiency in mice (11). That paper was the first report suggesting that miRNAs can be transferred between distinct animal species through dietary means. Our discoveries were corroborated in a recent report by investigators from the NIH-supported Genboree database who detected numerous dietary miRNAs in 6.8 billion sequencing reads from 528 human samples (12). Note that mammalian miRNAs are encapsulated in extracellular vesicles such as exosomes, thereby confer protection against degradation (13-16) and a pathway for cellular uptake by endocytosis (17, 18). Studies by us and an independent laboratory suggest that human and rat intestinal cells transport cow's milk

exosomes by endocytosis (Wolf, 2015, unpublished materials) and that milk exosomes may cross the intestinal mucosa without re-packaging in mice and enter circulation in intact form (19).

Here we tested the hypothesis that human endothelial cells transport cow's milk exosomes by a carrier-mediated process similar to the mechanism reported for the uptake of exosomes in intestinal cells. The uptake of dietary exosomes into endothelial cells is an important step in regulation of genes in peripheral tissues by dietary miRNAs in humans.

## Materials and methods

### Exosomes isolation and characterization

Cow's milk (1% fat) was obtained from a local grocery store in Lincoln, Nebraska. Milk was centrifuged at 12,000 x *g* at 4°C for 30 minutes to remove somatic cells and debris. Fat-free supernatant was mixed with an equal volume of 0.25 M EDTA (pH 7.0) and incubated on ice for 15 minutes to precipitate casein (20). The suspension was ultracentrifuged at 80,000 x *g* at 4°C for 60 minutes (Sorvall WX Ultra 80, F37L-8x100 rotor; Thermo Scientific, USA) to remove precipitated protein, milk fat globules, and microvesicles larger than exosomes. Exosomes were precipitated by centrifugation at 120,000 x *g* at 4°C for 90 minutes. The exosome pellet was re-suspended in sterile phosphate-buffered saline and filtered through a 0.22-μm membrane filter (Milex). Sodium azide was added to produce a final concentration of 0.01% and exosomes were stored at 4°C for up to 5 days.

Exosome purity and absence of aggregation was assessed as recommended by the International Society for Extracellular Vesicles (21). Briefly, whole protein extract from exosomes were resolved by gel electrophoresis (10 µg protein/lane) as described previously (22) and membranes were probed using mouse anti-bovine CD63 (AbD Serotec, UK) as a marker for exosomes, rabbit anti-serum to bovine alpha s1-casein as a marker for the species of exosome origin, and goat anti-bovine histone H3(Santa Cruz Biotechnology, USA) as a negative control (all at 1,000-fold dilutions). Bands were visualized using an Odyssey infrared imaging system (Licor, Inc.) and IRDye 800CW-labeled secondary antibodies (50,000-fold dilution). Anti-bovine alpha s1-casein was raised in rabbits (Cocalico, Inc, USA) using AHSMKEGIHAQQKEPMIGVGC coupled to keyhole limpet hemocyanin through amidated C-terminal and acetylated N-terminus as described previously (23). The anti-serum, but not pre-immunization serum, produced bands of the expected size with cow's milk and the synthetic peptide antigen, but did not react with human breast milk (**Online Supplementary Fig. 1**). Absence of exosome aggregation in our preparations was confirmed using negative staining transmission electron microscopy (Hitachi H7500, Japan) in the Microscopy Core Facility in the University of Nebraska-Lincoln. ImageJ (http://imagej.nih.gov/ij/index.html) was used to analyze the particle size of exosomes.

**Fluorophore conjugation**

Exosomes were labeled with FM-464 (Molecular Probes) as described previously (24). Unbound fluorophore was removed by pelleting the exosomes at 120,000 x g for 90 minutes, followed by three wash and ultracentrifugation cycles with sterile phosphate-buffered saline. The concentration of exosome protein was measured using a Nanodrop-

1000 spectrophotometer (NanoDrop Technologies, Inc., USA) and diluted into F-12K media at the desired protein concentration.

**Cell culture**

Human umbilical vein endothelial cells (HUVEC, passages 38-45) were purchased from American Type Culture Collection (CRL-1730) and cultured in F-12K medium, supplemented with 0.04 mg/ml endothelial cell growth supplement (Sigma), 0.1 mg/ml heparin (Sigma), 10,000 units of penicillin, 10 mg of streptomycin and 10% exosomes-free fetal bovine serum in a humidified atmosphere at 5% CO2 and 37° C. Exosome-free fetal bovine serum was prepared by sonicating the serum in a water bath for 1 hour, which destroyed most of the marker miRNA present in FBS (**Online Supplementary Fig. 2**). Media was replaced with fresh media every 48 hours.

**Transport studies**

In a typical experiment, $15x10^3$ HUVECs were seeded per well in a 96-well plate and allowed to adhere overnight. . Fluorophore-labeled exosomes were added to the wells to produce the desired concentration of exosome protein. Cells were incubated for the length of time denoted in Results. Media were removed and cells were washed three times with sterile PBS to remove extracellular exosomes. Controls were prepared by washing the cells immediately after addition of exosomes. Cell fluorescence (560/645 nm) was measured in a microplate spectrophotometer (BioTek, USA). Cells were harvested using trypsin and counted using a hemocytometer. Units of fluorescence were converted into mass of exosome transported by labeling a known mass of exosomes (protein) with fluorophore, and quantifying the fluorescence after removing unbound fluorophore. In select experiments, we measured the effects of the following treatments

on exosome transport: 1) cells were treated with 5 or 10 µg/mL of endocytosis inhibitor cytochalasin D (Gibco) for 30 minutes before adding exosomes (25); 2) cells were treated with 150 mM carbohydrate competitors D-glucose or D-galactose for 30 minutes before adding exosomes (26); and exosomes were treated with 100 µg/mL of proteinase K at 37°C for 30 minutes to remove surface proteins (18). All assays were performed in triplicate in at least three independent experiments. Transport kinetics were modeled using the Michaelis-Menten equation and non-linear regression; modeling was conducted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

**Statistical Analysis**

Means±standard deviations are reported. Homogeneity of variances among groups confirmed using Bartlett's test (27, 28). Statistical significance of differences among treatment groups was assessed using one-way ANOVA and Tukey-Kramer post hoc test. Analyses were performed in GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Differences were considered significant if $p<0.05$.

**Results**

Our exosome purification protocol yielded preparations of non-aggregated extracellular vesicles that were primarily composed of exosomes. When protein extracts were probed with anti-CD63 or anti-bovine alpha s1-casein strong bands were observed in western blots; in contrast, when protein extracts were probed with anti-histone H3 (negative control) no band was visible (Fig. 1A). The particle suspension was largely free of aggregates and the shape and contour of exosomes suggested vesicle integrity (Fig. 1B) with the particle size of 69±19.5 in diameter as expected for exosomes (29).

The uptake of milk exosomes into HUVECs is a carrier-mediated process. First, we established that exosomes uptake was linear with time up to one hour if 70 µg exosome protein was added to 200 µL media (Figure 2A), i.e. concentration below transporter saturation (see below). Subsequent transport studies were carried out using an incubation time of one hour. Exosome uptake followed Michaelis-Menten kinetics (Figure 2B): $V_{max}$=0.057±0.004 ng exosome protein x 40,000 cells$^{-1}$ x hour$^{-1}$ and $K_m$= 18.0±3.84 µg exosome protein/200 µL media. Exosomes uptake depended on the incubation temperature (Fig. 3). When a 5-fold excess (equaling to 6 times $K_m$) of unlabeled exosomes as added to the cell cultures, the uptake of fluorophore-conjugated exosomes decreased to 16.83±0.07 % of control ($P$<0.05, n=3 biological replicates each measured in triplicates). When cells were treated with 5 or 10 µg/ml, exosome uptake decreased to 63.5±21.3% and 40.8±22.0%, respectively, of controls, consistent with endocytosis.

Surface proteins played an important role in facilitating exosome uptake into HUVECs. When exosomal surface proteins were removed by treatment with proteinase K, exosome uptake decreased to about 50% of controls (Fig. 4A). The carbohydrate competitor galactose, but not glucose, caused a significant decrease in exosome uptake (Fig. 4B).

**Discussion**

Evidence is accumulating in support of the theory that dietary miRNAs may cross the intestinal mucosa and has biological activity in humans (11, 12, 19, 30). However, the mechanisms of intestinal transport and subsequent delivery to tissues are unknown. To

the best of our knowledge, this is the first paper to propose that the transport of cow's milk exosomes across vascular endothelial cells is mediated by endocytosis and that proteins on the surface of milk exosomes are compatible with proteins on the surface of human vascular endothelial cells. This study further corroborates the notion that dietary miRNAs have biological activity in humans.

This study has far-reaching implications for human nutrition and health. The National Cancer Institute defines bioactive compounds as "*a type of chemical found in small amounts in plants and certain foods […]. Bioactive compounds have actions in the body that may promote good health. They are being studied in the prevention of […] diseases"* (31). Milk miRNAs meet that definition, based on our previous studies (11). Future studies will need to reveal the extent to which dietary miRNAS contribute to the total miRNA body pool.

miRNAs have been implicated in virtually all aspects of human health and disease including bone health (32, 33), female and male reproduction (34, 35), arthritis and inflammatory bowel disease (36-38), metabolic syndrome (39-42), and cancer (43). Note that miRNAs also may have effects detrimental to human health. For example, the plasma concentrations of miR-210 are significantly higher in patients with pancreatic cancer compared with healthy controls (44), plasma miR-141 and miR-25 are elevated in prostate cancer and esophageal squamous cell carcinoma, respectively (45); plasma miR-21 is elevated in various types of cancer (46); and the urinary excretion of miR-126 and miR-182 is greater in bladder cancer patients compared with healthy controls (47).

Additional areas of health relevance include the possible use of milk exosomes as vehicle for the oral delivery of unstable drugs, and the potential role of dietary miRNAs

as confounders in biomarker studies that rely on miRNAs in body fluids. Evidence suggests that that dietary preferences affect miRNA signatures in human plasma (48).

Some uncertainties remain and will need to be addressed in future studies. First, the identities of the glycoproteins that mediates endocytosis of milk exosomes in endothelial cells is unknown and is an area of investigation in our laboratory. Second, it is possible that an excess of endogenous exosomes might compete with the endocytosis of dietary exosomes. We cannot assess this possibility until the plasma concentration of dietary exosomes has been established; distinct glycoproteome profiles on the surfaces of cells from distinct tissues might be a confounder in such studies. Third, our studies did not formally exclude the remote possibility that adherence to cells, rather than uptake into cells, accounted for cell fluorescence. We consider the mere adhesion of exosomes to cell surfaces an unlikely scenario, based on previous studies suggesting that milk exosomes may cross the intestinal mucosa without re-packaging in mice and enter circulation in intact form (19). Also, our own studies of intestinal transport of milk exosomes in human and rat cell cultures suggest that exosomes are endocytosed at the apical membrane for subsequent secretion across the basolateral membrane. Fourth, it is unknown whether dietary exosomes from species remotely related will be recognized by surface proteins in human cells.

We conclude that this study provides an important mechanistic framework for future studies of dietary extracellular vesicles and the roles of dietary miRNAs in human health and disease. In particular, due to the controversy surrounding the bioavailability of plant-borne miRNAs in humans, resources need to be devoted to this promising field of

research. A representative example would be the identification and validation of molecular signatures for assessing the dietary intake of vesicles and their cargo.

## ABBREVIATIONS USED

miR, microRNA; miRNA, microRNA; HUVEC, human umbilical vein endotelial cells

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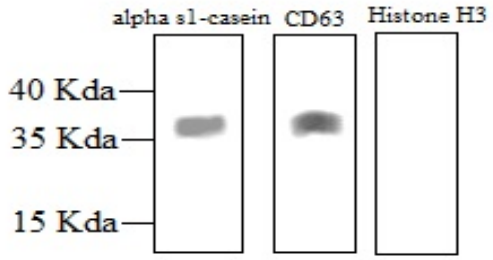
**A)** **B)**



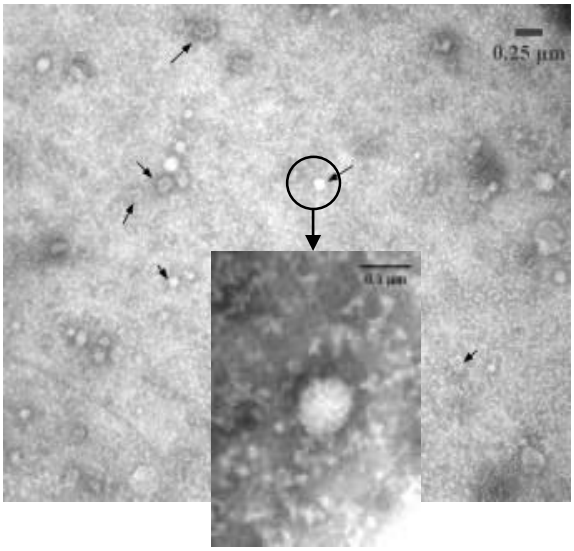




FIGURE 2.1. *A*: western blot result showing the absence of intercellular protein histone H3 but positive for commonly enriched exosomal protein; CD63 and bovine protein origin; alpha s1-casein. *B*: electron microscopy images of cow's milk exosomes showing round shape (69±19.5 nm) and intact vesicles.

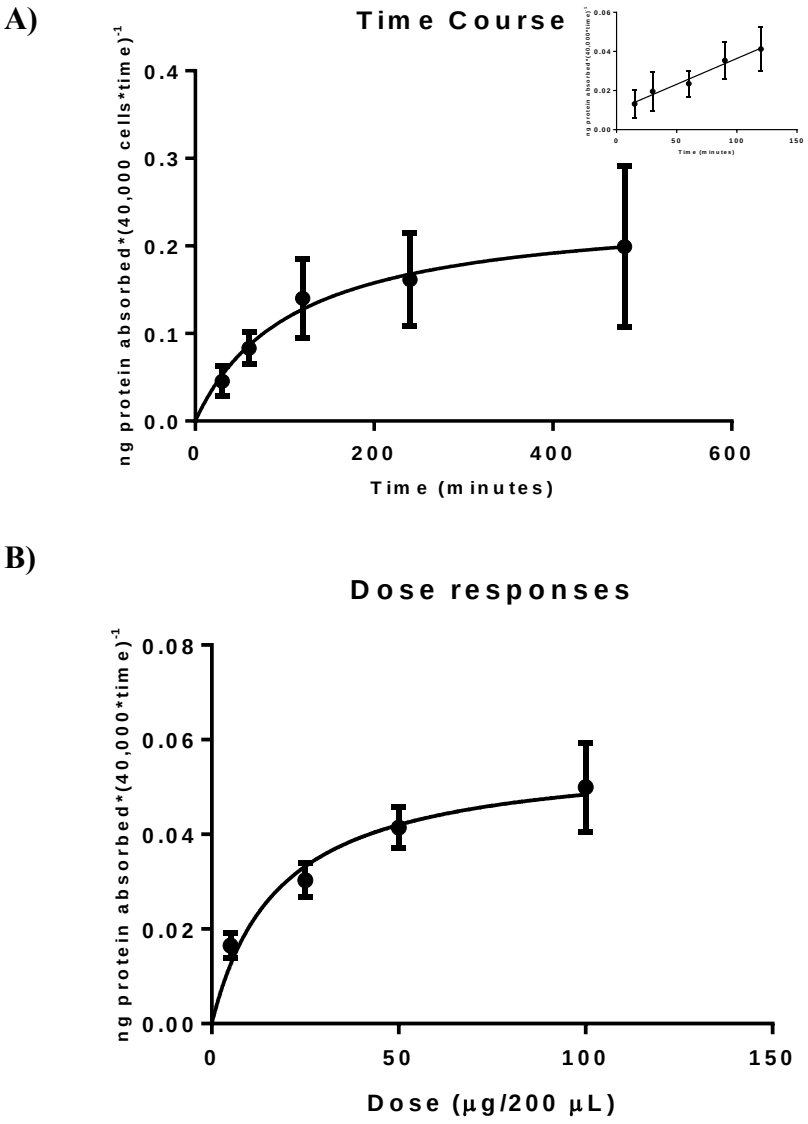



FIGURE 2.2. Milk exosomes uptake is time (A) and dose (B) dependent (n=9, 3 independent replicates).

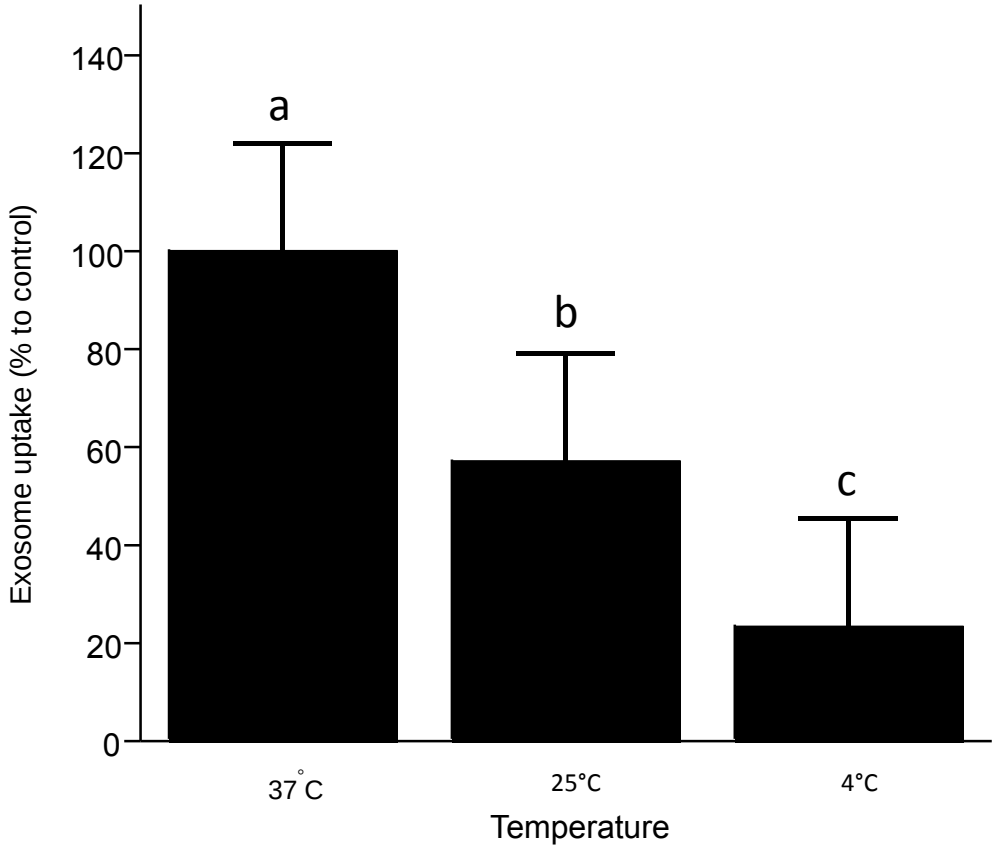



FIGURE 2.3. Reducing temperature to room temperature and 4°C significantly reduced exosomes uptake (n=9 from 3 biological replicates; $p<0.05$).

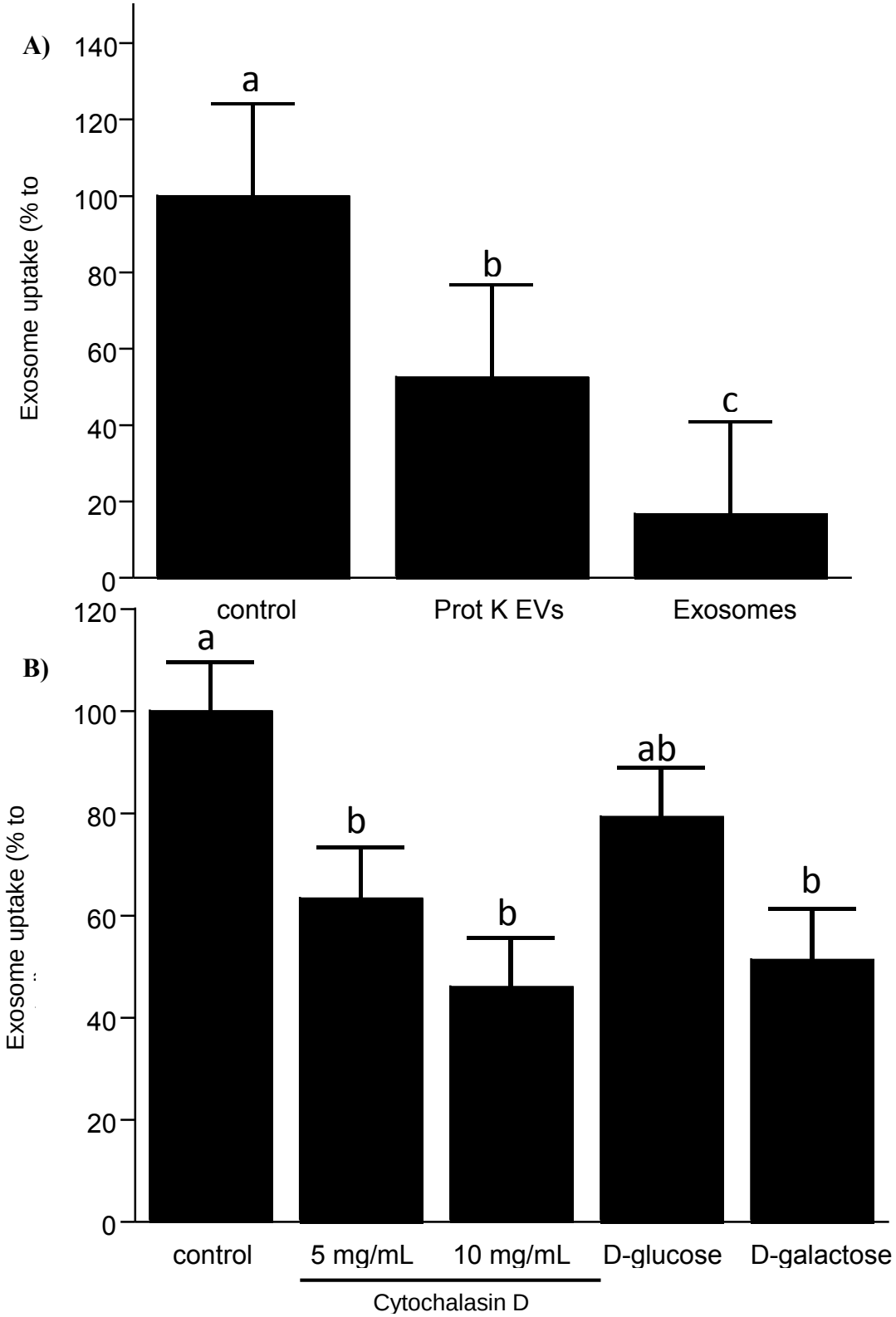



FIGURE 2.4. Treatment of exosomes with proteinase K (Prot K) and addition of unlabeled exosomes (Exosome) (A) as well endocytosis inhibitors (B) significantly reduced milk exosome uptake (n=9, 3 independent experiments, $p<0.05$).

# Chapter 3

# Loss of miRNAs during Processing and Storage of Cows (*Bos taurus*) Milk

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Running head: Loss of miRNAs during processing and storage of milk

**ABSTRACT**

MicroRNAs (miRs, miRNAs) play central roles in gene regulation. Previously, we reported that miRNAs from pasteurized, store-bought bovine milk have biological activity in humans. Here we assessed the effects of milk processing, storage, somatic cell content, and handling by consumers on the degradation of miRNAs in milk; we also quantified miRNAs in dairy products. Pasteurization and homogenization caused a 63% loss of miR-200c, whereas a 67% loss observed for miR-29b was statistically significant only in skim milk. Effects of cold storage and somatic cell content were quantitatively minor (<2% loss). Heating in the microwave caused a 40% loss of miR-29b but no loss of miR-200c. Milk fat content had no effect on miRNA stability during storage and microwave heating. The concentrations of miRNAs in dairy products were considerably lower than in store-bought milk. We conclude that processing of milk by dairies and handling by consumers causes a significant loss of miRNAs.

**Key words**: heating, MiRNAs, milk, processing, storage

## INTRODUCTION

MicroRNAs (miRs, miRNAs) are small non-coding RNAs that play essential roles in the regulation of genes at the posttranscriptional level in plants and animals.[1] Mature miRNAs are about 22 nucleotides long and bind to complementary sequences in the 3'-untranslated region of mRNAs. Perfect or near perfect base pairing of the miRNAs and its target mRNAs typically results in mRNAs degradation, whereas less perfect base pairing typically results in inhibition of mRNAs translation.[2-3] Traditionally, miRNAs have been considered endogenous regulators of genes, i.e., miRNAs synthesized by a given host regulate the expression of genes in that host. Recently, our laboratory refuted this paradigm. We provided strong evidence that 1) humans absorb biologically meaningful amounts of miRNAs from nutritionally relevant doses of cow's milk, 2) physiological concentrations of milk miRNAs affect human gene expression *in vivo* and in cell cultures, and 3) endogenous synthesis of miRNAs does not compensate for dietary miRNA deficiency in mice.[4] Our discoveries were largely modeled on miR-29b and miR-200c, but likely hold true for all miRNAs encapsulated in milk exosomes.[5-6] To the best of our knowledge, our previous paper is the first to provide unambiguous evidence that miRNAs can be transferred between distinct species through dietary means. In contrast, previous claims that miRNAs from plants affect human gene expression[7-8] are highly controversial and were met with skepticism by the scientific community.[4, 9-12] Based on the above observations, milk miRNAs are a novel class of bioactive food compounds as defined by the National Cancer Institute in the United States.[13] The discovery that milk miRNAs are bioactive food compounds has broader implications as miRNAs play essential roles in gene regulation,[2-3] cell communication,[14-15] and human health.[16-23]

This study focused on determining the effect of milk processing, storage, somatic cell content, and handling by consumers on two miRNAs, miR-29b and miR-200c levels based on the following rationale. In bovine milk, miR-29b and miR-200c are among the most abundant miRNAs.[20] MiR-29b is an important regulator of bone mineralization in humans, as it increases osteoblast differentiation[16] and decreases osteoclast differentiation and function.[17] MiR-200c decreases cancer risk by targeting the transcription factor ZEB1, which induces E-cadherin expression, thereby limiting epithelial-to-mesenchymal transition, a key event in metastasis.[24-25] Also, the nucleotide sequences of miR-29b and miR-200c in bovine milk are identical to those of their human orthologs.[26] Our rationale for including the somatic cell count in our analysis was to assess whether an increase in milk cells, as seen in mastitis, might be a confounder in the analysis of milk miRNAs.

In Western societies, the majority of milk is processed prior to consumption. In fact, the production and sale of raw milk dairy products is illegal in many states in the United States and pasteurization is required.[27] Moreover, while the per capita consumption of milk has declined from 236 pounds in 1982 to 195 pounds in 2012, total dairy consumption increased by 11% during the same time period.[28] Therefore, we considered it worthwhile to assess the effects of processing on the miRNA content in both milk and dairy products.

Little is known about the effects of processing and storage on milk miRNAs levels. In two studies, synthetic miRNAs were added to bovine milk and their stability after exposure to harsh treatments such as acid and RNase was assessed and compared to the stability of endogenous miRNAs in milk.[6, 20] Synthetic miRNAs were rapidly degraded, whereas endogenous miRNAs were resistant to treatment. However, the harsh treatments

applied in these studies are not representative of the treatments applied in commercial dairy production. In this study, we assessed the effects of pasteurization, fat content, cold storage, heating as well as processing into dairy products on content of milk miRNAs.

## MATERIALS AND METHODS

**Chemicals.** Guanidinium thiocyanate and ethanol were purchased for use in the NucleoSpin miRNA plasma RNA extraction kit (Macherey-Nagel Inc., Bethlehem, PA). TRIzol was purchased from Life Technologies (Grand Island, NY).

**Milk and dairy products.** Raw, whole, 2%, and skim cow's (*Bos taurus*) milk was obtained from The Pennsylvania State University Creamery (University Park, PA) from separate collections in three consecutive weeks in May 2014. All milk was procured from the Penn State Animal Science Department's Holsteins breed herd. The milk for this study was processed from using raw milk and cream routinely supplied to the Penn State Berkey Creamery, University Park, PA and stored under intermittent agitation in a 22,712.5 liter raw milk silo at 2.2°C (Feldmeier Equipment, E-015-05; Little Falls, NY). The milk contained 3.25% milk fat and 8.9% milk solids non-fat (near-infrared method, CEM, Turbo Smart5, Model 907990; Matthews, NC).

For the preparation of the product, milk (3.25% Milk Fat; 12.15% Milk Solids Non Fat) was pasteurized at 75.55°C with a 28-s holding time (APV Paraflow, Serial number 20053003000302; Goldsboro, NC). It was homogenized at 145 Bar and 60°C (APV Gaulin Homogenizer, Serial Number 20052410702; Lake Mills, WI). The product was standardized using a Westfalia Separator, type MSE 55-01-177; Oelde, Germany. A details and process diagram for preparation of whole cow's milk is presented in figure 1.

After cooling the product was transferred to a 7200 liter refrigerated storage tank at 2.2°C (Feldmeier Equipment, E-015-05; Little Falls, NY). The product was bottled on filling machine (Federal Manufacturing, Serial Number 1/12.4GL843; Milwaukee, WI) and stored in a conventional cold-milk warehouse at 3.0°C.

On our initial collection dates, milk of all fat levels were stored at 4ºC for up to 15 days, and aliquots were taken and frozen at -80ºC every other day. In a separate experiment bovine cells were removed from raw milk by centrifugation (500 g, 10 min, 4°C) to determine whether somatic cells are a meaningful confounder when analyzing the concentrations of miRNAs in milk from healthy cows. Samples were frozen at -80°C and shipped on dry ice to Lincoln, NE, for miRNA analysis. Samples from all fat levels of milk on day 15 were heated in the microwave for 15 seconds and analyzed after cooling off to room temperature. Dairy products other than milk were purchased from grocery stores in Lincoln, NE. All samples were produced and analyzed as biological repeats in triplicate.

**MiRNA analysis.** Milk samples were spiked with a synthetic internal standard (twenty-five attomoles) prior to extraction of miRNAs using miSPIKE Synthetic RNA (IDT Technologies).[4] Dairy products (100 mg) other than milk were extracted using TRIzol prior to addition of the synthetic internal standard. MiR-29b and miR-200c were quantified using quantitative real-time PCR as described previously.[4]

**Statistics.** Analysis by Bartlett's Test Homogeneity suggested that variances were homogeneous.[29] The paired t-test was used for pairwise comparisons. One-way analysis of variance (ANOVA) and Fisher's protected least significant differences were used when comparing more than two groups. Repeated measures ANOVA was used for assessing the effects of storage time on miRNA concentration. StatView 5.0.1 (SAS

Institute; Cary, NC) was used for conducting statistical analyses. Means ± SD are reported. Differences were considered statistically significant if $P \leq 0.05$.

**RESULTS**

Pasteurization and homogenization of raw milk resulted in a 63±28% decrease of miR-200c in whole milk; effects were similar for 2% fat milk and skim milk (**Fig. 3.1A**). The effect was less pronounced for miR-29b for which a significant decrease (67±18%) was observed only in skim milk (**Fig. 3.1B**). Cold storage of milk did not affect the concentration of mir-29b and miR-200c in whole milk, 2% milk and skim milk up to 15 days; 2% fat milk is shown as a representative example in **Fig. 3.2.** Somatic cells are not meaningful confounds regarding the analysis of miRNAs in milk from healthy cows. When somatic cells were removed from raw milk by centrifugation and analyzed for miRNA content, the cellular miRNAs were found to contribute less than 2% of the total miRNAs present in raw milk before centrifugation: 1.1±0.9% for miR-29b and 0.14±0.08% for miR-200c.

Processing in the household has the potential to cause a considerable loss of some miRNAs in milk. For example, the concentration of miR-29b decreased by 40±28% when processed milk was heated in the microwave and cooled to room temperature compared to milk before heating (**Fig. 3.3**). In contrast, when milk was heated in the microwave the concentration of miR-200c was not statistically different compared with unheated controls.

The concentration of miRNAs varied considerably among the product tested (Table 3.1), but were generally lower than the concentration in pasteurized whole milk (compare

to figure 3.2). Fresco queso dip was a notable exception and contained higher concentrations of miRNAs than those observed in milk.

## DISCUSSION

In a recent paper we reported the importance of milk miRNAs for gene regulation in humans.[4] That report has major implications for the roles of milk and possibly other dairy products in human health. Cow's milk contains meaningful quantities of 245 miRNAs,[20, 30] and 71.4% of these miRNAs are predicted to target about 11,000 human transcripts (unpublished observations). In addition to the roles of miR-29b and miR-200c in bone health and cancer prevention,[16-17, 24-25] respectively, miRNAs have been implicated in various aspects of human health and disease including hypertension, insulin resistance and diabetes, hyperlipidemia and atherosclerosis, reproduction, immune function and Crohn's disease.[18, 20-21, 31-33]

We propose that milk has a meaningful effect on human health, mediated by miRNA-dependent gene regulation. The potential importance of dietary milk miRNA intake is supported by data suggesting that 1) Americans consume large quantities of milk and dairy products,[28] 2) a large proportion of milk miRNAs is encapsulated in extracellular vesicles, thereby providing protection against degradation [5-6] and a pathway for cellular uptake by endocytosis,[34-35], and 3) milk miRNAs are resistant against degradation during storage (this study).

The concentrations of miRNAs varied considerably among the dairy products tested (**Table 1**), but were generally lower than the concentrations in pasteurized whole milk

(compare **Fig. 2**). Fresco Queso Dip was a notable exception and contained higher concentrations of miRNAs than those observed in milk.

Our previous studies of milk miRNAs in humans and mice were conducted using 1% fat milk from the grocery store.[4] Based on this study, the content of miRNAs is about two times higher in unprocessed milk compared with pasteurized, store-bought milk. Note that we have no intent recommending the consumption of raw cow's milk by humans, because of food safety concerns associated with raw milk. We observed that a loss of milk miRNAs occurred only during pasteurization, homogenization, and processing to dairy products. This observation is consistent with previous studies of milk miRNAs. For example, endogenous miRNAs were not degraded when milk was exposed to harsh treatments such as low pH or treatment with RNase.[6, 20] It is reasonable to propose that encapsulation of miRNAs in extracellular vesicles[5] prevents miRNA degradation, based on the following lines of evidence. 1) When synthetic miRNAs are added to milk and subjected to low pH or RNase treatment, the miRNAs are rapidly degraded.[6, 20] 2) When exosome membranes in milk were disrupted by sonication for preparing miRNA-depleted mouse diets in previous studies, miR-29b was rapidly degraded to concentrations below detection limit.[4] Presumably, degradation was due to milk RNases gaining access to miRNAs released from exosomes. 3) When milk was fermented to produce yoghurt, miRNA concentrations decreased to levels much lower than in milk (this study). We speculate that the decrease was due to the lysis of exosomes during fermentation and the large amounts of RNases produced by microbes. 4) When milk was homogenized, miRNA concentrations decreased by on average 50% (this study). We speculate that the decrease was caused by a disruption of exosome mebranes by shear forces applied during

homogenization. Collectively, our studies suggest that milk, and perhaps dairy products, have the potential to contribute to the miRNA body pool in humans.

Some uncertainties remain to be addressed in future studies. For example, this study was modeled based on miR-29b and miR-200c, however, there is a possibility that distinct miRNAs may be differentially metabolized.[8, 36] Another layer of uncertainty is the possible effects of feeding regimens, season, and breed on the miRNA content in milk. Moreover, while this study suggests that somatic cells in milk from healthy cows do not contribute meaningful amounts to the total miRNA content in milk, it is possible that the increased somatic cell count in milk from cows suffering from mastitis[37] may cause an artificial increase in milk miRNA concentrations. Our previous studies suggest that plasma miRNA concentrations decrease by 61% in mice fed a milk miRNA-depleted diet for four weeks. This observation is consistent with milk miRNAs contributing meaningful quantities to the miRNA body pool, but does not necessarily establish the essentiality of dietary miRNA intake. Clearly, this is an uncertainty that will need to be addressed in future studies. Finally, it is conceivable that miRNAs from foods other than milk also contribute toward the total body pool of miRNAs.

**ABBREVIATIONS USED**

miR, microRNA; miRNA, microRNA

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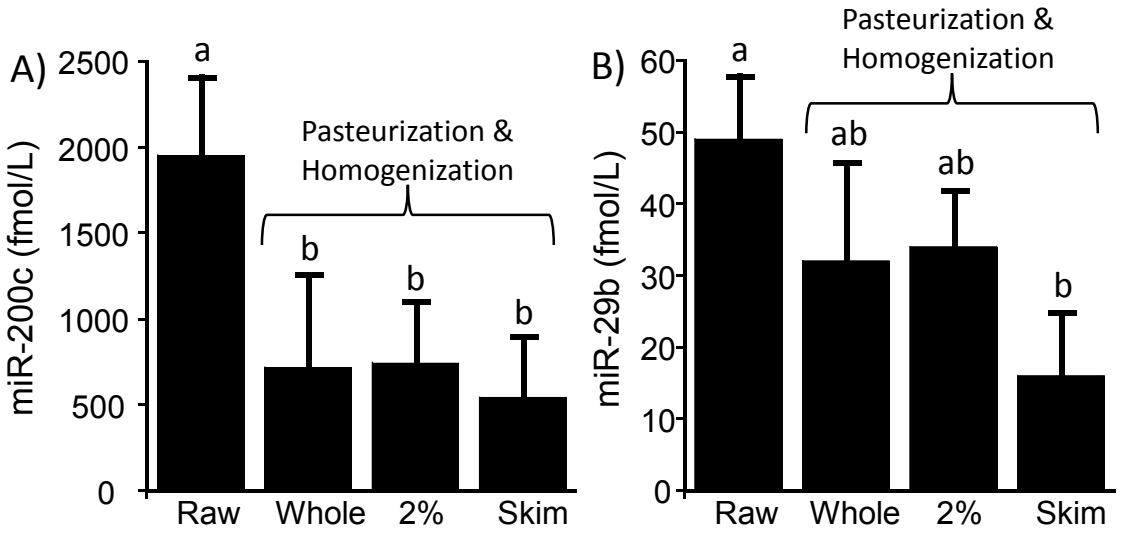


**Figure 3.1.** Loss of miR-200c (A) and miR-29b (B) during milk pasteurization and homogenization of milk with different fat content. [a,b]Significantly different (n=3 biological replicates, $P<0.05$).

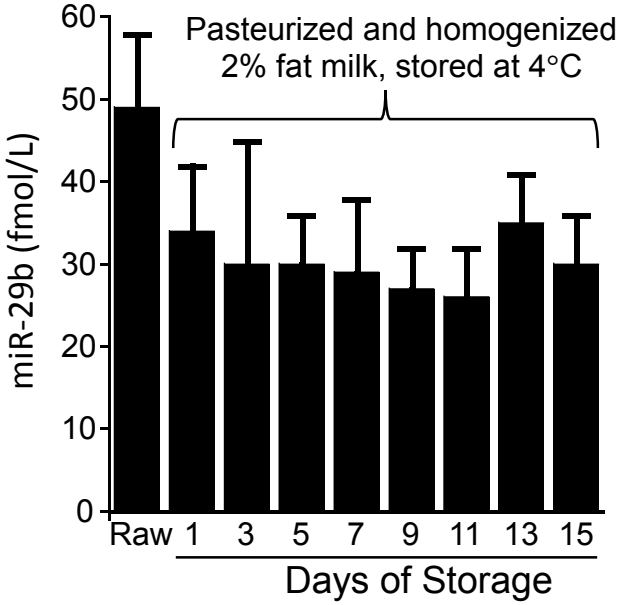


**Figure 3.2:** Storage at 4°C did not affect the concentrations of miR-29b in pasteurized and homogenized 2% fat milk (n=3 biological replicates, $P>0.05$).

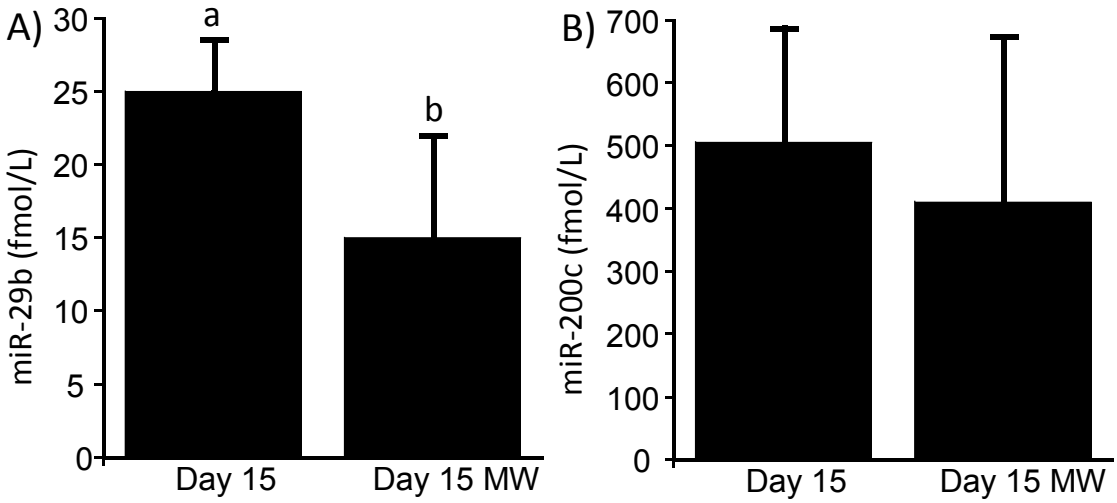


**Figure 3.3:** Loss of miR-29b (A) and miR-200c (B) during heating of whole milk in the microwave after 15 days of storage at 4°C. Abbreviation: MW, microwaved.

[a,b]Significantly different (n=3 biological replicates, $P<0.05$).

**Table 3.1. Concentration of miRNAs-29b and -200c in dairy products**

| | MiRNA | |
|---|---|---|
| **Product** | **miR-29b** | **miR-200c** |
| | *fmol/kg* | |
| Best Choice Yogurt | 0.9±0.10 | 37.6±2.8 |
| Fresco Queso Dip | 36.1±5.5 | 1029.8±478.6 |
| Greek Yogurt | 14.2±3.9 | 462.3±126.9 |
| Half and Half | 3.0±0.17 | 513.3±159.2 |
| Heavy Whip Cream | 2.6±1.3 | 342.0±132.9 |
| Parmesan Cheese | 4.9±1.9 | 232.0±64.5 |
| Upstate Farm Yogurt | 2.4±1.0 | 216.9±93.8 |

Data are means±SD, n=3.

# Chapter 4

# Future Study

Although there is still a debate whether miRNA can be transferred across species (1-3), our lab has provided an evidence that miRNA indeed can be transported across species (4, 5). It is hypothesized that the miRNA transport across species was mediated by exosomes or other extracellular vesicles as these small vesicles protect the miRNA from enzymatic degradation and harsh environment such as low pH (6, 7). Furthermore, exosomes also facilitated the transport into the recipient cells as numerous studies have reported the ability of the cells to internalize exosomes and use the miRNA cargo inside exosomes to promote function (5, 8-12).

In recent study, we provide another evidence that cow's milk exosomes can be transported into human endothelial cells through endocytosis process. However, several endocytosis pathway warrant further study. For instance, we only provide the evidence that the transport was mediated by actin-dependent process. Actin is required for most endocytosis process including phagocytosis, macropinocytosis and chlatrin-mediated endocytosis (13-15). This indicated the non-specific endocytosis mechanism that mediated the uptake of cow's milk exosomes into human endothelial cells. Therefore, it is necessary to address this issue by performing another inhibitory studies or protein inhibition through RNA interference, specific for each endocytosis mechanism, to answer specific endocytosis-mechanism.

Study suggest that surface glycoprotein in exosomes play an important role in cellular recognition and internalization of exosomes by target cells (16-18). In this study we suggest that glycoprotein surface in cow's milk exosomes is important for mediating the uptake into human endothelial cells as indicated by lower uptake of cow's milk exosomes in the presence of galactose and when the milk exosomes was treated with

proteinase K. It is interesting if we could provide the glycoprotein class or component in surface area of cow's milk exosomes that mediated the uptake process in human endothelial cells. Thus, performing another transport study using surface-blunt glycoprotein of milk exosome is important in the future to characterize the glycoproteins that mediates the uptake in human cell. Additional mass-spectrophotometry (MS) to identify the surface glycoprotein in cow's milk exosome can also be used to support the data derived from the transport study using surface-blunt glycoprotein of cow's milk exosomes.

In addition, we only based our experiment by indirect observations. It is still not clear whether the exosomes from cow's milk is indeed internalized by endocytosis, not by fusion with plasma membrane. Electron microscopy image or phase contrast microscopy should be performed in the future study to clarify the data obtained by the FM4-64 labelled exosomes.

Recent study suggests that cow's milk exosome can be transported across the rat's intestinal cells without being degraded or repackaged by the intestinal cells (19). However, question remains whether this event occur in human peripheral cells and the miRNAs present in cow's milk exosomes can promote function after being transported across endothelial cells. Transwell study is a way to answer this question. In the first experiment, HUVEC were grown in the apical surface and cow's milk exosomes were added when HUVECs have formed monolayer in the apical surface. Exosomes was isolated from the apical and basolateral surfaces of transwell by using differential centrifugation method. Electron microscopy, western blot and Q-RT-PCR for selected miRNAs were performed to characterize the exosomes isolated from both of the surfaces.

In the second experiment, HUVECs were grown in the apical surface of transwell and stable transfected HEK-293 carrying the miR-200c reporting gene were grown in the basolateral of transwell. Milk exosomes was added and luciferase activity was measured after incubation for several hours.

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# Appendix

qRT-PCR primers used to quantify gene expression

| **microRNA** | **Forward Primer Sequence (5-3')** | **Reverse Primer Sequence (3-5')** |
|---|---|---|
| miSpike | CTCAGGATGGCGGAGCGGTCT | Universal primer mix |
| miR-29b | GTAGCACCATTTGAATCAGTGTT | Universal primer mix |
| miR-200c | TAATACTGCCGGGTAATGATGGA | Universal primer mix |